Peripheral Blood Mononuclear Cells Express Mutated NCCT mRNA in Gitelman’s Syndrome: Evidence for Abnormal Thiazide-Sensitive NaCl Cotransport

NATALIA ABULADZE,* NORIMOTO YANAGAWA,† IVAN LEE,* OAK D. JO,† DEBRA NEWMAN,* JAMES HWANG,* KOICHI UYEMURA,‡ ALEXANDER PUSHKIN,* ROBERT L. MODLIN,‡ and IRA KURTZ*  
*Division of Nephrology, Center for Health Sciences, †Division of Nephrology, Sepulveda Veterans Administration, and ‡Division of Dermatology, UCLA School of Medicine, Los Angeles, California.

Abstract. Genetic analysis has demonstrated complete linkage between the human thiazide-sensitive sodium chloride cotransporter gene (NCCT or TSC) and Gitelman’s syndrome (GS). Several genomic NCCT mutations have been reported. This study was performed to determine whether peripheral blood mononuclear cells (PBMC) express NCCT mRNA and whether defective PBMC NaCl cotransport could be demonstrated in GS. PBMC were isolated from two brothers with GS, their parents, and healthy control subjects. Northern analysis demonstrated that PBMC express NCCT mRNA and whether defective PBMC NaCl cotransport could be demonstrated in GS. PBMC were isolated from two brothers with GS, their parents, and healthy control subjects. Northern analysis revealed that NCCT mRNA is expressed in PBMC. The sequence of full-length NCCT cDNA amplified from normal PBMC was identical to human renal NCCT cDNA. Two different mutations were detected in the patients’ NCCT cDNA (compound heterozygote). In cDNA derived from the patient’s paternal allele, exon 24 was deleted, resulting in a premature stop codon (after amino acid 920). cDNA derived from the patient’s paternal allele had an additional 119-bp insertion between exons 3 and 4, generating a premature stop codon (after amino acid 187). The patient’s genomic DNA had a previously described 5’ splice site mutation in intron 24, GGT → GTT (maternal allele), and a new 3’ splice site mutation in intron 3, CAG → CAA (paternal allele), which resulted in the activation of a nearby cryptic splice site in intron 3. The latter mutation was not present in 300 normal chromosomes. To determine the functional significance of these findings, chlorothiazide-inhibitable 22Na uptake was measured in PBMC from control subjects, the parents, and the patients with GS in the presence of bumetanide. In control PBMC, chlorothiazide inhibited 22Na uptake by approximately 9%. PBMC from the two patients with GS failed to respond to chlorothiazide. These results demonstrate that PBMC can be used for mutational analysis of NCCT mRNA in patients with GS. Furthermore, functional evidence is provided that the underlying cause of GS is defective NCCT NaCl cotransport.

Gitelman’s syndrome (GS) is a disorder characterized by hypokalemia; hypomagnesemia; renal potassium and magnesium wasting; chloride-resistant metabolic alkalosis; hypocalciuria; normal BP; and elevated plasma renin and aldosterone levels (1,2). Recently, Simon et al. reported complete linkage between GS and the thiazide-sensitive cotransporter (NCCT) locus (SLC12A3) on chromosome 16q13, and specified GS as an autosomal recessive disorder with 99% penetrance (3). These findings have subsequently been confirmed by other groups (4–8). In situ hybridization and immunocytochemistry studies, combined with functional data, indicate that the NCCT gene encodes the apical membrane thiazide-sensitive NaCl cotransporter (NCCT, also known as TSC) in the distal convoluted tubule (9–12). In the rat kidney, polyclonal antibodies against rTSC1 intensely label the apical membrane of the early distal convoluted tubule, with less intense staining of the late portion terminating within the connecting tubule (12). In addition to the distal convoluted tubule, there is evidence for NCCT expression in osteoblast-like cells (13).

Simon et al. identified a wide variety of mutations distributed throughout the NCCT gene consistent with loss-of-function alleles (3). Additional potential loss-of-function mutations have recently been reported (6,14,15). Missense mutations are most frequent and are more frequently localized to the intracellular domains of the protein rather than the extracellular or transmembrane domains. Many of the patients described thus far are compound heterozygotes. This finding, together with the documentation of independent mutant alleles within the same kindred, suggests that mutant alleles are not rare. In the Swedish and Italian populations, the prevalence of heterozygotes based on phenotypic expression is approximately 1% (3,16,17). Simon et al. have estimated a sporadic prevalence of 0.001 and a mutant allele frequency of 1 in 200 (3).

The effect of specific mutations on the molecular and functional properties of the transporter in patients with GS has been difficult to study, because of the localization of NCCT expression to the renal distal convoluted tubule. In this study, we...
demonstrate that NCCT mRNA is expressed in peripheral blood mononuclear cells (PBMC) obtained from healthy control subjects and two brothers with GS. For mutational analysis, PBMC were used as an easily obtainable source of NCCT mRNA to complement the results from genomic DNA sequencing. Additional studies were performed to determine whether PBMC could be used to assess NCCT function in healthy control subjects and in patients with GS.

Materials and Methods

Clinical Features of GS Kindred

A kindred was identified with clinical features of GS (Figure 1). Two of the three offspring of a nonconsanguineous marriage had hypokalemia, hypomagnesemia, renal potassium and magnesium wasting, chloride-resistant metabolic alkalosis, hypocalciuria, and normal BP values. The propositus was a 42-yr-old man who presented to UCLA with a history of chronic hypokalemia and hypomagnesemia. His medical history was unremarkable except for occasional cramps in his calves. He had no history of laxative or diuretic abuse or of vomiting, and he was not taking any medications. The patient’s 47-yr-old brother also had a history of chronic hypokalemia and hypomagnesemia, and intermittent muscle cramps. Both parents and a third brother are asymptomatic and have no electrolyte abnormalities. There was no previous history of laxative/diuretic abuse or vomiting. Laboratory values in the propositus were: 136 mEq/L serum sodium; 2.4 mEq/L potassium; 94 mEq/L chloride; 28 mEq/L total CO₂; 10 mg/dl blood urea nitrogen; venous blood gas, pH 7.47; 38 mmHg Pco₂; 0.7 mg/dl creatinine; 1.25 mmol/L ionized calcium; 9.2 mg/dl total calcium; 3.2 mg/dl phosphorus; 1.1 mg/dl magnesium; 16.6 ng/ml per h supine plasma renin; 168 pg/ml supine aldosterone; 13 pg/ml parathyroid hormone; and 42 pg/ml 1,25 vitamin D. Urine chemistries were: 64 mEq/L sodium; 42 mEq/L potassium; 58 mEq/L chloride; 1 mg/dl calcium; 4.3 mg/dl magnesium; 67.4 mg/dl phosphorus; and 81 mg/dl creatinine. A diuretic screen was negative. The 47-yr-old brother with GS had very similar laboratory values.

Isolation of PBMC

PBMC were isolated from the two patients with GS, the parents, and from healthy control subjects as follows: 15 ml of heparanized blood was added to 25 ml of phosphate-buffered saline to which 12 ml of Ficoll-Hypaque was then added. The cells were centrifuged at 2100 rpm for 20 min in a Sorvall RT 6000B centrifuge, and the supernatant was discarded. The cell layer was resuspended in RPMI 1640 + glutamine + streptomycin/penicillin. The cells were then centrifuged at 1600 rpm for 20 min, and the pellet was resuspended in RPMI a second time.

Northern Analysis

Total RNA was isolated from PBMC and human kidney using the method of Chomczynski and Sacchi (18). Poly(A)⁺ was isolated from PBMC, with the PolyATract mRNA isolation system (Promega, Madison, WI). The RNA was separated on a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane. An NCCT-specific 1.7-kb PCR product (from human PBMC NCCT cDNA) was generated with the following primers: (sense) GACCAGCTGTACCCACTGATC, and (antisense) GAGCTGTGGACAGGAGATGTC.
The probe was random-primed with \( [^{32}P] dCTP \) to a specific activity of approximately \( 1.5 \times 10^9 \) dpm/\( \mu \)g. The filter was prehybridized at 42°C for 2 h, using 50% formamide, 6× saline-sodium phosphate-ethylenediaminetetra-acetic acid, 0.5% sodium dodecyl sulfate (SDS), Denhardt's solution, and 0.1 mg/ml sheared herrings testes denatured DNA. After prehybridization, the filters were incubated with the \( ^{32}P \) probe, using 25 ml of hybridization buffer. The probe was denatured and added to the hybridization solution at \( 10^7 \) dpm/ml. The filter was probed at 42°C for 18 h and washed in 1× SSC, 0.1% SDS at 45°C for 60 min (3 changes, 350 ml per wash); after exposure for 6.5 h, the filter was rewashed in 0.1× SSC, 0.1% SDS at 65°C for 60 min. The actin DNA was T4 PNK labeled with \( [^{32}P] \gamma \) ATP to a specific activity of 2.5 μCi/pmol. The filter was prehybridized at 42°C for 2 h, using 50% formamide, 6× saline-sodium phosphate-ethylenediaminetetra-acetic acid, 0.5% SDS, Denhardt's solution, and 0.1 mg/ml sheared herrings testes denatured DNA. After the prehybridization, the filter was probed with the \( ^{32}P \) probe, using 25 ml of hybe solution at 0.5 pmol/ml. The filter was probed at 42°C for 18 h and then washed 3 times in 1× SSC, 0.1% SDS at 45°C for 30 min.

**Analysis of NCCT Genomic DNA and cDNA**

Genomic DNA was isolated from the parents, both patients with GS, and healthy control subjects, using the Turbo Gen DNA purification kit (Invitrogen). We were unable to obtain DNA from the unaffected sibling. All 26 exons of the NCCT gene were amplified using primers described by Simon et al. (3). The genomic DNA was sequenced using an ABI 310 automated sequencer (Perkin Elmer). Total RNA from PBMC was obtained from both patients with GS, and healthy control subjects. The total RNA was reverse-transcribed with avian myeloblastosis virus reverse transcriptase. Full-length NCCT cDNA from both patients with GS and healthy control subjects was amplified and sequenced. In both patients, NCCT cDNA derived from the paternal and maternal alleles revealed two different mutations (see Results). In the region of the paternal allele mutation (exons 3 and 4), the following primers were used to amplify cDNA from both patients with GS and healthy control subjects: (sense) CAGGCAGACAATGCAGAACT, (antisense) CCAATGATGCGGATGTCG; (nested, (sense) GTGCGCTCCGCTGTTGTT; (antisense) GACTTGACCTTGGCAATG. In the region of the maternal allele mutation (exons 23 to 25), the following primers were used to amplify cDNA from both patients with GS and healthy control subjects: (sense) CGTGTGGTCGGTAGCAGCCAG, (antisense) AGGCTTCAGCAGGCCAGCATG. The genomic DNA NCCT sequence in 150 healthy control subjects (300 chromosomes) was compared with the sequence abnormalities in the patients.

**Assessment of NCCT Function**

\( ^{22} \)Na uptake was measured in PBMC from the patients, the parents, and healthy control subjects (n = 6). PBMC were washed and resuspended in a solution containing (in mM): 140 NaCl, 2.5 K₂HPO₄, 1 CaCl₂, 1 MgCl₂, 5 Hepes, pH 7.4 (solution A). Aliquots of the cell suspension (3 × 10⁶ cells/100 μl per tube) were placed in 12×75-mm polystyrene tubes and preincubated at 37°C in a shaking water bath for 10 min with bumetanide (0.1 mM) to inhibit the NaK2Cl cotransporter. Isotope uptake was initiated at 37°C by adding 100 μl of solution A containing \( ^{22} \)Na (5 μCi/ml, carrier-free, Dupont New England Nuclear, Boston, MA) with bumetanide (0.1 mM).
Tracer uptake was linear for 2 min and was measured in the presence and absence of chlorothiazide (1 mM). $^{22}$Na uptake was terminated after 1 min by the addition of ice-cold isosmotic stop solution containing (in mM): 140 tetramethylammonium hydroxide, 140 gluconic acid lactone, 2.5 $K_2$HPO$_4$, 7 Ca gluconate, 2 Mg gluconate, 5 Heps, pH 7.4 (solution B). The cells were pelleted at 2400 rpm for 5 min at $4^\circ$C (GS-6 refrigerated centrifuge, Beckman Instruments, Fullerton, CA). After an additional cycle of pelleting and washing, the cells were lysed by adding 1 ml of 0.2N NaOH to each tube and heated at 75$^\circ$C in a heating block for 10 min. Aliquots of cell lysate were transferred to 10 ml of scintillation fluid (Ultima-Gold, Packard, Downer’s Grove, IL), and the radioactivity was quantified by scintillation counting (1600-TR, Packard). The remaining cell lysates were assayed for protein using Coomassie brilliant blue G250 with bovine serum albumin as the standard (19). Separate samples of cells (which underwent the same uptake procedure after adding 1 ml of 12% trichloroacetic acid per tube and boiling for 5 min) were used as blanks and subtracted from the total uptake.

Materials
The following were used in this study: chlorothiazide and bumetanide (Sigma, St. Louis, MO); PolyATract mRNA isolation system (Promega); avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis, IN); Ficoll-Hypaque (Pharmacia, Piscataway, NJ), Turbo Gen DNA purification kit (Invitrogen, Carlsbad, CA).

Results
Mutational Analysis
As shown in Figure 2, human PBMC express an approximately 4.5-kb NCCT transcript. Full-length NCCT cDNA from both affected siblings was sequenced. In addition, genomic DNA from both parents and the two affected siblings was isolated, and all NCCT exons were sequenced. The results obtained from propositus are shown because the sequence of both affected siblings was identical. As shown in Figures 3 and 4, the patient's genomic DNA had a new 3' splice site mutation in intron 3, CAG $\rightarrow$ CAA (paternal allele), and a previously described 5' splice site mutation in intron 24, GGT $\rightarrow$ GTT (maternal allele) (3). The results of the mutational analysis of the patient's cDNA are shown in Figures 5 and 6. cDNA derived from the paternal allele had an additional 119-bp insertion between exons 3 and 4, generating a predicted premature stop codon (after amino acid 187). Intron 3 was completely sequenced, and a cryptic splice site CAG was found 121 bp prior to the upstream intron 3/exon 4 boundary. This site was activated in the patient's paternal allele, resulting in the 119-bp insertion of intronic DNA prior to exon 4 (Figure 6). In cDNA derived from the patient's maternal allele, exon 24 was deleted, resulting in a predicted premature stop codon after amino acid 920 (Figure 6).

Functional Analysis
Additional experiments were done to determine whether NCCT function was abnormal in the two brothers with GS. The NaCl cotransporter function was assayed by measuring the chlorothiazide-inhibitable $^{22}$Na uptake in PBMC obtained from the patients with GS, each parent (heterozygote carriers), and healthy control subjects. As shown in Figure 7B, chlorothiazide significantly decreased $^{22}$Na uptake in PBMC from...
Figure 5. (A) PCR amplification of NCCT cDNA from PBMC (exons 3 and 4), using the following primers: (sense) CAGCGGACATGGGCAGAAC, (antisense) CCAATGATGCGGATGTCGTT; nested, (sense) GTGGCCTTCGGCTGGGTC, (antisense) GACTTGACCTTGCACTTG. Lane 1, 1-kb ladder; lane 2, normal PBMC; lane 3, patient with GS. Unlike control cDNA, two bands, 320 bp (upper) and 201 bp (lower) were detected in the patient with GS. The sequence of the upper band revealed a 119-bp intronic insertion prior to the beginning of exon 4. (B) PCR amplification of NCCT cDNA from PBMC (exons 23 to 25), using the following primers: (sense) CGTGTGTTCGTAGCGGCCAG, (antisense) AGGGTCTCCAGCCAGCCCATG. Lane 1, 1-kb ladder; lane 2, normal PBMC; lane 3, patient with GS. Unlike control cDNA, two bands, 407 bp (upper) and 271 bp (lower), were detected in the patient with GS. The sequence of the lower band revealed a deletion of exon 24. Both patients with GS had identical findings; therefore, the results of one patient are depicted in Panels A and B.

healthy control subjects and the parents, but had no effect on PBMC from the patients with GS.

Discussion

Simon et al. first demonstrated complete linkage of GS to the NCCT gene (SLC12A3) on chromosome 16q13, and specified GS as an autosomal recessive disorder with 99% penetrance (3). The linkage to chromosome 16q13 has been confirmed by other groups (4–8). A wide variety of genomic mutations were found to be distributed throughout the NCCT gene consistent with loss-of-function alleles (3). Additional potential loss-of-function mutations have recently been reported (6,14,15). Missense mutations are most frequent and are more often localized to the intracellular domains of the protein rather than the extracellular or transmembrane domains. Many of the patients described thus far are compound heterozygotes. In the present study, we have shown that NCCT mRNA is expressed in PBMC. The finding that PBMC express NCCT mRNA permitted a mutational analysis at both the genomic and mRNA level. This was especially useful given the impracticality of obtaining renal tissue for the patients for mutational analysis at the mRNA level. The 5' splice site mutation in intron 24 previously reported by Simon et al. (3) in the patients maternal allele was shown to result in the deletion of exon 24 in NCCT mRNA. A new 3' splice site mutation resulted in the activation of an upstream cryptic splice site and insertion of a 119-bp intronic fragment prior to exon 4 in NCCT mRNA derived from the paternal allele.

The effect of genomic splice site mutations on subsequent RNA editing cannot be predicted with certainty, i.e., insertion of intronic sequence versus exon deletion (20). Therefore, we analyzed NCCT cDNA in the two affected patients. The results demonstrate that both mutations would be predicted to generate truncated NCCT protein. The functional consequence of these mutations was studied. PBMC isolated from the patients had no demonstrable thiazide-inhibitable 22Na uptake. We have recently demonstrated (unpublished observations) that PBMC also express NKCC2 mRNA. Therefore, the methodology used in this study may be equally useful in patients with Bartter's syndrome (21).

The patients characterized in the present study were compound heterozygotes, and therefore the absence of PBMC thiazide-sensitive NaCl cotransport was a result of two different loss-of-function mutations. It would be more difficult using PBMC from compound heterozygous patients to study the effect of a given missense mutation that does not cause complete loss of function. The expression of mutated NCCT mRNA in heterologous expression systems would be required
Figure 6. Sequence of NCCT cDNA (numbered according to GenBank). (A) Two different cDNA sequences were detected in the patient. cDNA derived from the paternal allele (1-F) had a 119-bp insertion prior to the beginning of exon 4. The sequence of the cDNA from the maternal allele (2-M) was normal in this region. (B) Two different cDNA sequences were detected in the patient. The sequence of the cDNA from the paternal allele (1-F) was normal in this region. cDNA derived from the maternal allele (2-M) had a deletion of exon 24.

**A**

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In heterozygote carriers, although the serum and urine electrolytes are within the normal range, one might speculate that these individuals have subtle but detectable salt and potassium wasting associated with decreased urinary calcium excretion. In addition, it would also be of interest to determine whether subtle differences in PBMC thiazide-sensitive NaCl cotransport can be detected. The measured PBMC thiazide-inhibitable 22Na uptake in the parents of the two patients was not decreased. Whether upregulation of the NCCT mRNA/protein derived from the normal allele in each parent accounts for these findings requires further study.

Previous studies have shown that NCCT is expressed in the mammalian renal cortex (10), osteoblast-like cells (13), and at low levels in small intestine, prostate, colon, and spleen (7). The results of the present study provide the first evidence for expression of full-length NCCT in human nonepithelial tissues. Furthermore, NCCT was shown to mediate a component of sodium transport in PBMC. The role this transporter plays in
uptake was measured in the presence and absence of chlorothiazide (1 mM) and bumetanide (0.1 mM). (A) Time course of PBMC $^{22}\text{Na}$ uptake.

**Figure 7.** Effect of chlorothiazide on sodium transport in PBMC. Chlorothiazide-inhibitable $^{22}\text{Na}$ uptake was measured in the presence of bumetanide (0.1 mM). Each data point represents the mean of four measurements. (B) $^{22}\text{Na}$ uptake was measured in the presence and absence of chlorothiazide (1 mM). Chlorothiazide decreased $^{22}\text{Na}$ uptake in the control group ($n = 6$) by approximately 9% ($p < 0.05$) and failed to significantly alter transport in PBMC obtained from the two patients with GS. Chlorothiazide-inhibitable $^{22}\text{Na}$ uptake in the parents (heterozygote carriers) was similar to the control group.

**Acknowledgments**

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